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RECENT INVESTIGATIONS INTO THE PHENOMENON
OF HAEMOLYSIS.

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Thesis by *Eric Ponder, M.B.*

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INTRODUCTION.

In this essay an attempt is made to collect into a short space, researches which have a bearing on haemolysis, and to correllate them one with another.

The essay is prefaced by a consideration of the structure of the erythrocyte; and is thereafter divided into parts dealing with various aspects of haemolysis. The part dealing with complement-amboceptor systems is necessarily brief, since our present knowledge is very small; the same remarks applies to the part dealing with animal haemolysins. However undesirable such a division of ~~the~~ essay into parts may be, it is necessary, in order to give any clear conception of recent work. The parts are sub-divided into sections, numbered in Roman numerals, so as to facilitate cross reference.

Nearly all ~~the~~ mathematical work has been placed in a separate part at the end of the essay.

A full bibliography is given. References to the bibliography in the text are made by means of ordinary figures adjoining the name of the investigator.

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A. THE STRUCTURE OF THE ERYTHROCYTE.

I. The erythrocyte is a circular biconcave disc containing haemoglobin. Certain observers have denied that the biconcavity exists in circulating cells. Some claim that while circulating they are cup-shaped (1); recently it has been suggested that they are discs. (2) The biconcavity, however, is usually accepted. Hartridge points out that this form is a very suitable one, for only in a sphere and in an infinitely thin disc could gasses reach the centre with equal readiness. The sphere being undesirable because of its small surface, and the infinitely thin disc form impossible, the red cell may be looked upon as an ideal compromise, the edges of the disc being thickened to prevent the peripheral parts being reached more readily than the centre. (3).

II. The average size of the erythrocyte in man is 7.8μ in diameter, and varying between 2μ at their thickest part, and 1.5μ at their thinnest part. Price Jones (4) states that they vary in size during the day, increasing with exercise and decreasing with rest: according to him the variation in diameter may amount to 0.6μ . This writer made his measurements chiefly on stained films: he checked them/

them on fresh preparations. Ponder (27) has shown his results to be mathematically unreliable; and even impossible, since if this occurred, nearly half the cells in the body would be haemolysed. Slight variations in the depth of the cell, however, are constantly occurring, and these may take place without the membrane being subjected to any extension. (LIV.). The biconcave shape, and also the depth of the cell, will depend on the relative external and internal osmotic pressures; the osmotic pressure of serum has been shown to be persistently higher than that of the cell (VII.), consequently the biconcavity would be explained. By treating the erythrocyte as a compound of three ¹elipsoids of rotation about minor axes, Ponder has calculated the volume under various conditions: the ~~the~~ details are given in section LIV. It may be taken that the average volume of the erythrocyte is about 71.0 cubic μ , while the weight of a single erythrocyte will be 72.0×10^{-6} grammes.

III. There are at least three views of the structure of the red cell. The first is that of Schafer (5), that the red cell is an envelope composed of lipoids for the most part, and containing a solution of haemoglobin. The second is that originally advanced by Rollett, and recently/

recently revived by several workers whose experiments are alluded to later,—(14,15,55), that the red cell consists of a cell-stroma. Some observers, while not going the length of postulating a cell stroma, consider that the interior of the erythrocyte is occupied by a network, such as described by Golgi (6), or by "supporting bands", as described by Emrys-Roberts (7). The third view is that of Stewart, who considers that the cell contains within its lipoid membrane, a gel, holding haemoglobin in its meshes: this view must not be confused with that of Rollett. It is unnecessary to present the wellknown arguments of the various investigations, but one or two points must be noted.

- (I) The view of Rollett is an absurdity. A disc-shaped body of any isotropous substance, by imbibing water, could never become spherical.
- (II) The statement that the erythrocyte owes its disc like shape to the fact that it is surrounded by lipoid is no better. A lipoid body takes this shape because it is subject to external surface tension stresses. If it be made to double its volume, by, for instance, water diffusing into it, it will still tend to keep a flat shape, for the external/

external stresses will still be present as before. Under no conditions would a sphere result. The red cell, however, becomes, in hypotonic saline, a sphere; consequently one may conclude that its original flat shape is due to the fact that it is surrounded by a lipoid membrane of a disc-like shape, and that this shape is not due to surface tension, but is structural.

(III) The assumption that the red cell is surrounded by an envelope of a disc-like shape is an agreement with its behaviour on water diffusing into it. The view of Schafer is confirmed by mathematical considerations (section LIV seg.)

(IV) The view of Stewart is also reasonable. The "gel" which is supposed to exist inside the erythrocyte is not a structure of a definite nature, but merely a physical state in which the haemoglobin exists. All the phenomena occurring when osmosis takes place would be quite possible, provided the elastic envelope be admitted.

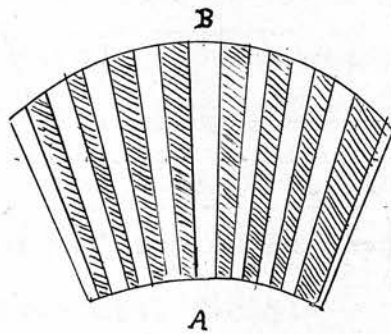
Diffusion/

Diffusion through a gel is nearly the same as diffusion through water; so, as far as changes of shape, volume, etc., under different osmotic changes, are concerned, the gel-filled envelope would behave in the same way as an envelope filled with a simple solution. Haemoglobin must be in colloidal solution, from the size of its molecule; the idea of a gel, under certain circumstances, accordingly seems reasonable. If the contents of the cell were diluted, as occurs when the erythrocyte is placed in hypotonic saline, this gel might readily become fluid. Any destruction of the membrane would also effect this.

IV. Beechhold (8) considers that the envelope is composed of a fine network of protein, in the meshes of which lecithin and cholesterol are held, thus giving a lipoid envelope on a protein framework. The envelope is to be considered, on the average, as uniform in thickness and elasticity. It is obviously not simply a fatty membrane, as indicated by Norris (9), for it is wetted by water, diffusion taking place freely through it. It may be noted that lecithin is wetted by water. (10). The idea of the cell wall consisting of several phases has been developed/

developed considerably. Schryver (11) has done very ingenious experiments, to elucidate the structure of the envelope. He considers that it is composed of a system of lipoids, proteins, etc., held together by a gel, similar to chocolate gels. Such gels are attacked by various substances, in the same order as the order of Overton's narcotic effects on cells. Schryver therefore suggests that substances may attack this gel which he considers to be a component of the cell wall, thus weakening the wall and producing haemolysis. Unfortunately, his researches were carried out only with substances such as chloroform, ether, alcohol, etc. A similar conception is due to Robertson (12), who, reasoning from ultramicroscopic appearances, considers the cell wall as a mixture of lipoids and other substances, in which the lipoids ^{are} oriented in a special way, so that the lipid particles be radially, forming funnel shaped pores, (Diagram 1.) so that water-soluble substances could pass in, but with difficulty pass out, owing to the almost imbroken fatty nature of the internal wall: such a conception would explain the semipermeable nature of the envelope to some substances, and its impermeability to others (VIII.).

Free/



I. DIAGRAM TO ILLUSTRATE ROBERTSON'S HYPOTHESIS.

A.... INTERIOR OF CELL.

B.... EXTERIOR OF CELL.

SHADED PARTS.... RADIIALLY ARRANGED NON-AQUEOUS
PHASE.

Free (13) has also suggested that the envelope of cells may be made up of several phases, some more watery than others; anything affecting the phase distribution would then affect the cell. The idea of a lipid membrane is altogether rejected by v. Knaffl-Lenz, who considers the membrane to be composed of protein: he further, however, believes that a stroma exists.(14.)

V. Much of the statements regarding the nature of both the cell in general, and the membrane surrounding it, are very uncertain, being based more on analogy or theoretical considerations than on experimental evidence. A description of the structure of the erythrocyte must, however, contain the following demonstrated facts:-

- (1) That the erythrocyte has an elastic envelope, containing lipoids and proteins, and wetted by water.
- (2) That the cell owes its shape to the structure of its envelope, and not to external surface tension forces.
- (3) That the interior of the cell contains either a fluid or a substance with similar dynamic properties, for example, a thin jelly.

B. HAEMOLYSIS CONSIDERED AS A RESULT OF OSMOSIS
AND CELL PERMEABILITY.

VI. It has long been recognised that the envelope of the erythrocyte acts as a semi-permeable membrane. Consequently if the cell, the contents of which have an osmotic pressure corresponding to $\Delta = .550$ (in man), be placed in a fluid of a lower osmotic pressure, this fluid will diffuse into the cell, distending it and causing bursting. The factors controlling this diffusion are, however, very complex; simple diffusion laws do not hold.

VII. In the first place, there is a constant difference between the osmotic pressure inside the cell, and that outside it, in the serum. The difference amounts to about 400 m.m. of mercury - a not inconsiderable difference. This was shown by Hamburger. (15), who by freezing, thawing, and compressing red cells with sand, obtained a fluid, whose osmotic pressure was lower than that of serum. He regarded the probable cause of this as an adsorption of the electrolytes by the "cell stroma": it might be/

be suggested with greater reason that the adsorbent was the sand. The extensive researches of Collip (16), settled the matter beyond dispute. Collip did not extract the cells with sand, but obtained them by centrifuging. He found that the Δ values of serum and cells varied according to the species. The following table gives some of his results:

	Δ Serum	Δ Cells
Man	0.585	0.520
Sheep	0.621	0.582
Rabbit	0.615	0.570
Dog	0.647	0.581
Cat	0.68	0.605
Pig	0.609	0.575

In every case Δ for cells is lower than Δ for serum. From the value of Δ the osmotic pressure may be found by the formula,

$$P = \frac{\Delta}{1.87} \times 17,000$$

where P is the osmotic pressure in m.m. of mercury.
The/

The average difference is given by Collip as about 400 m.m. of mercury. Collip throws some doubt on his own results by calculating, from osmotic pressure readings the percentage composition of the cell as regards fluid, for he finds that if a volume of red cells be diluted with water, the following formula holds,

$$\Delta_1 = \left[\frac{100 + x}{x} \right] \Delta_2.$$

where x is the percentage of fluid in the cells, and where Δ_1 and Δ_2 are the depressions of freezing point for undiluted and diluted cells respectively. He finds that $x = 75$ approximately: the following values being obtained:-

	x
Man	76.5
Sheep	76.0
Rabbit	72.7
Dog	77.6
Cat	72.0
Pig	72.0
Fowl	64.0

If/

If these results be true, the interior of the erythrocyte cannot be all fluid: but, as Collip remarks, depressions of freezing point under the conditions of experiment may not be a true guide to osmotic pressure. In the present state of our knowledge, however, it must be accepted that the osmotic pressure within the cell is less than that outside. This is supported by the fact that the red cells are poorer conductors than serum, an observation which has been made by many observers. The biconcave shape of the erythrocyte would be explained by this difference of osmotic pressure.

VIII. The cell membrane is a peculiar one in that it allows certain substances to diffuse and not others. This may be due to something in its internal structure as suggested by Robertson (IV). Even proteins may pass through the membrane, for isotonic saline removes them from the cell (Scott, 17), and proteins introduced into the blood pass into the erythrocytes. (Manwaring and Yoshio, 18). In human cells, sugars diffuse, according to Kozawa (19), but not in the cells of the goat, rabbit or puppy. Brinkman (20) states that this diffusion does not occur until the first stages of coagulation have set in. The cell is permeable to anions, as demonstrated by Hamburger (21), who contradicts Gürber's statement that Na/

. Na and K do not diffuse.

IX. The reader will naturally observe that experiments regarding diffusion, if made in the usual way, though parchment or collodion membranes, may be fallacious, as the wall of the red cell contains a large percentage of lipoids. Fousneau and Vulquin made special membranes with the object of avoiding this fallacy. The membranes used by them were made of collodion containing castor oil and lipoids: they found that a collodion tube containing over 2 per cent of oil was impermeable to water, but if lecèthin was added the membrane became permeable. The addition of cholesterol did not affect the membrane in any way. (22).

X. Further, it has been shown by Stiles and Kidd (23) that if a salt diffuse into an animal cell, the final equilibrium is not constant, but depends on the external concentration, the ration being given by the adsorbtion equation. It appears then that as well as diffusion taking place through the membrane, some of the solute is adsorbed at the interface.

XI. The membrane has also been shown to be polarized. Gerard (24) made potential measurements in/

in both plant cells and in erythrocytes and came to this conclusion: extensive researches by Kosaki and Seki confirm his results. These observers investigated the charge on red cells by studying their migration towards the poles of a circuit, when suspended in various electrolytes. They found that acids, and monovalent salts caused migration to the anode: that di-valent salts were neutral in their effects, whereas alkalis caused migration to the cathode. They concluded that strongly charged cell membranes are most rapidly haemolysed by alkalis. (25).

XII. From these considerations, it will be seen that the investigation of osmosis through red cell envelopes is not a simple matter. The degree of permeability depends much on the type of substance which diffuses. To begin with cell penetration by acids will be considered. Crozier (26) has investigated the relative ability of acids to pass through cell membranes, using the mantle of chomodosia zebra as a basis. This tissue turns red at $P_H = 5.6$. Crozier estimated the time taken for various concentrations of acids to penetrate. He found, in general, that the rate of penetration depended on (I) the ionisation of the acid (II) its concentration, and (III) its chemical nature: he concluded that the surface layer of the cell was acted on/

on chemically. Since his results are of importance, certain conclusions may be given. (a) In general monobasic acids penetrate less than dibasic acids, and these less than mineral acids. (b) strong acids penetrate in order of ionisation. (c) among weak acids the order of penetration is that of their surface tension on water. It has been shown that certain acids have a tendency to form "membrane" at the cell interface, the membrane preventing diffusion; monobasic acids do this most, and mineral acids least, so according to Crozier the rate of penetrability is just the reverse of that membrane production, as one might reasonably expect. It is doubtful how much this applies to Haemolysis, for Ponder (27) has shown that the order of intensity with which acids produce haemolysis is not that order with which they penetrate; although mineral acids are the most haemolytic acetic acid, which is the slowest cell penetrator, haemolyses rapidly. Neither is the order of penetrative power the order with which the acids accelerate haemolysis by saponin etc. (XXVI). Haas (28) has shown that the order in which plant cells are penetrated by acids is similar to the order for animal cells: he considers ionisation to be the determining factor. As has been mentioned the existence of a change on the cell surface would influence the activity of the acid. Loeb considers that acids act on the lipoids.

XIII. Much more complex than permeability to acids is the permeability/

permeability of the cell wall to various electrolytes. Certain salts have been shown to have a tendency to form "membranes". This property has been investigated by Clowes (29) with a dropping instrument; he dropped soap solutions through olive oil. To the soap solution various salts were added, these having the effect of dispersing to a greater or less extent the surface film. Such an alteration would produce a variation in the number of drops per minute; so that the drops would indicate the degree of dispersion of the film at the interface, or, what is the same thing, of its permeability. Clowes found that calcium especially promoted the formation of a "membrane", or in other words, decreased the dispersity of the surface layer. Sodium salts, he found, increased the dispersity, thus making the wall permeable. McArthur and Caldwell (30) independently found that lecethin and similar solutions are precipitated by calcium; such a precipitation would decrease the permeability of a cell wall, which contained lecethin, as does the envelope of the red cell. The observations of Clowes are borne out by many observers. Moore found that the permeability of the egg of the sea urchin was increased by NaCl, and KCl, but lessened by Mg. Sr. Ba and Ca, in accordance with Clowes results regarding/

regarding the effects of these salts on the dispersity of the surface layer.(31). Brooks, working with dandelion tissue, found that diffusion was increased by NaCl, and lessened by the presence of Calcium. In general, he concluded that monovalent kations increase permeability, and that di-valent and tri-valent ions diminish permeability. (32). McClendon, working on *Cassiopeia*, a jelly fish, found that Na, K and OH increase cell permeability, whereas Ca, and Mg inhibit diffusion. (33) Brinkman (34) has applied these results largely to haemolysis, and considers that the permeability of the red cell depends largely on the presence or absence of Ca. He accordingly uses a special fluid for suspending red cells.

XIV. The nature of this "membrane" is under some dispute.

While, according to some observers it may be due to a precipitation of the lipoids by certain ions, Loeb (35) suggests that it is due to a chemical combination with the cell wall components. Loeb found, experimenting with the eggs of *Fundulus*, that certain cell membranes are impermeable to certain ions, for example K, unless (a) a second salt be present or (b) K have time to combine with the proteins of the cell wall. This combination he terms "the salt effect", and is in reality, a change in the cell wall/

wall itself; it is a complex phenomenon, depending on (I) the nature of the salts concerned, and (II) the concentration of such salts. It is observable that this "salt effect" occurs only in the presence of acids or bases. Loeb attributes it to the fact that salts will unite with proteins in the presence of acids, or bases, but not in a neutral medium. Tadokow (36) suggested that the passage of certain ions coagulate the proteins of the envelope, thus forming a membrane which prevents diffusion. Thus the effect of certain ions is explained by some as an interference with the lipoids of the envelope, but by others as an interference with proteins.

XV. The relation between concentration and time taken for diffusion has been shown by Williams (37) to follow an logarithmic course: she finds the equation:-

$$\log T = R - A(\log C) + 1.$$

to describe the reaction: T being the time, and A a constant depending on the electrolyte. The influence of temperature has been studied in plant cells by Stiles. The van't Hoff law is found to hold fairly well, the coefficients varying from 1.5 to 3.8. (38).

XVI.

XVI. Various species of animal produce cells with a very different resistance to osmosis; for instance, the cells of the guinea pig is much more resistant than the sheep cells, the cells of the pig being intermediate. This is doubtless due to differences in the structure of the cell wall.

XVII. That haemolysis occurs as the result of osmosis alone seems undoubted. The action of the hypotonic saline is such an action: the degree of rapidity of the osmosis, which, again, depends on factors enumerated above. A very good example of osmotic pressure haemolysis is given by Kosakai (39). Red cells can be suspended without injury in 1 per cent boric acid. They are, however, instantly haemolysed by immersion in isotonic sugar, serum, or saline: this, presumably, is due to osmosis; for if put in concentrated saline or sugar, no haemolysis occurs, nor does it occur if isotonic saline be slowly added. Kosakai observes that the minimal non-haemolytic concentrations of saline, serum or sugar, are of identical osmotic concentrations. The fluid passing into the cell increases the cell volume, and stretches the envelope: when a certain stress is developed, the envelope bursts at its weakest point.

XVIII./

XVIII. It may be shown that, if the initial volume of the cell be about 70 cubic μ , that it may expand to a volume of about 80 cubic μ , without any stress on the envelope, owing to the biconcave shape of the latter. From this point onward the cell will become more and more nearly spherical, its circumference at first slightly shortening. It will eventually become a sphere, the radius of this sphere will increase until a body of about 300 cubic μ in capacity is reached: at this point the average cell bursts. Now even if a cell be haemolysed by a surface tension stress on the exterior, osmosis plays an important part; for if the cell be dragged out from outside, in order that it may yield to this pull its membrane must stretch; to allow this stretch the cell must increase in volume; for this to occur, the surrounding fluid must enter the cell. The speed at which the cell increases will be governed by the speed at which fluid can enter, together with the magnitude of the external stress. If the cell envelope be attacked by a chemical substance, or if it be weakened by being partly dissolved, the stress on the envelope will need to be less in order to produce the rupture.

These points are obvious, but are rarely stated clearly.

C. HAEMOLYSIS CONSIDERED AS A RESULT OF SURFACE-TENSION.

IXX. It is a well known fact that some of the most powerful haemolytic substances are those which are powerful lowerers of surface-tension, e.g. saponin, bile salts, or soaps. Their mode of action on the erythrocyte has been conceived as being a sudden production of a negative external pressure, and a continuation of this pressure, thus straining the envelope of the cell, and finally rupturing it. The point of action is supposed to be the cell interface. There has been some doubt regarding haemolysis by surface tension, however, in the minds of some workers, who have considered the effect of saponin, bile salts, etc., as a solubility effect: since these substances to some extent dissolve lipoids. For a believer in the stroma theory of the red cells, haemolysis by surface tension can mean nothing.

XX. Neuschloss (40) pointed out that certain electrolytes altered the surface tension of lecithin. Na antagonises K, and mixtures of K and Ca. By drawing up tables, this investigator found the necessary quantities of various electrolytes required to produce an equilibrium, i.e. a solution which did not alter the surface tension of lecithin. He then investigated/

investigated haemolysis by hypotonic solutions, and found that minimal haemolysis occurred in solutions which produced an equilibrium as regards surface-tension. These solutions cause the smallest change in surface tension on the envelope, therefore less dispersion of the envelope and a greater resistance. Bechhold, (41) whose view of the cell wall has been alluded to (IV), showed that the surface tension was at a maximum when the cell was in 0.85% NaCl. He considers that any change, especially lowering, of the concentration of the fluid will be followed by a change in surface tension - this will put stress on the envelope, and cause haemolysis, irrespective of osmosis. As pointed out in section XVIII, osmosis will however play a part, for it determines the rate at which the cell wall yields to the stress. This idea is borne out by researches of Furabata (42) who found that, if saponin haemolysis be produced in a viscid medium, haemolysis is slow; owing, it would appear, to the diffusion of the viscid substance being slow. Handovsky (43) also observed that if haemolysis by saponin be produced in a medium which would diffuse into the cell with difficulty, haemolysis was a slow process; neither of these two observers recognised the principle involved, however.

XXI. Flohr has studied the exact changes in surface tension produced by saponin on glass (44). Although it is probably/

probably fallacious to assume that the same changes take place at a lecithin interface, the knowledge of how saponin behaves in general is of use. Flohr performed his estimations by the dropping method, and investigated solutions of saponin from 1/8 per cent to 3 per cent.

Applying the formula

$$\frac{W \times S}{X} \times 73 \text{ ergs} = \gamma$$

- where W = the number of drops from the instrument if water be used, X = the number of drops in the case of the unknown, and S = the density of the unknown, - γ has been calculated from Flohr's figures by Ponder. The minimum value for γ is in 1 per cent saponin solution, more dilute solutions exert less surface-tension change. Since the dilutions in which saponin haemolysis is conveniently studied are 1 - 1000 to 1 - 40,000, Ponder has investigated the values of γ for these dilutions:-

<u>dilution</u>	<u>γ (ergs)</u>	<u>dilution</u>	<u>γ (ergs)</u>
1/1000	40.9703	1/10,000	54.4084
1/4000	45.9040	1/20,000	57.8051
1/5000	48.2843	1/30,000	61.2056

The value of γ for saline was 64.6056 ergs; all measurements were at 12°C, taking the value of S for saponin in these dilutions/

dilutions in saline as 1.0047, and using the capillary tube method and the formula,

$$\gamma = \frac{1}{2} h.r.g.s.$$

where $g = 981$ dynes.

The lowering of surface tension produced by these solutions is considerable.

XXII. If the conception of haemolysis outline in section XX be correct - that the yielding of the envelope to the surface tension strain is dependent on the permeability of the envelope, - one would expect that salts which render the envelope relatively impermeable would delay surface-tension haemolysis by saponin, etc. This was found by Horber (45) who finds that monovalent ions do not retard this haemolysis, but divalent ones, such as calcium, do. Horber could not explain this result.

XXIII. Woodward and Alsberg (46) deny that there is any relation between surface tension and haemolysis in the case of the saponins: since they investigated the surface tension of saponin solutions on glass, and not on lipoids, and since the technique of their haemolytic experiments was inadequate, their conclusions are not reliable.

It/

It is doubtless true, nevertheless, that in haemolysis by saponin other factors than surface-tension reduction plays a part. The influence of the permeability of the cell wall has been dealt with: further, saponin may attack the membrane, dissolving it, and so weakening it.

XXIV. Bile salts may certainly dissolve lipoids, and this may increase their haemolytic action. They also lower surface tension markedly, especially the taurocholate. Strong evidence of their haemolytic effect being primarily a surface tension one has been brought forward by Ponder (47), who showed that a strong solution of sodium glycocholate was much less haemolytic than a dilute solution, the optimum ^{um} dilution for haemolysis being about 1 in 1000: this is a strong argument against the view that the haemolysis is a result of solubility. The same observation applies to olein; pure olein is non-haemolytic, but a dilute solution produces rapid haemolysis.

XXV. Further strong evidence that the haemolytic action of bile salts, saponin, etc., is due to changes in surface tension which they produce is afforded by the researches of Katsche and Jaudas (48). These investigators noted that soaps, in non-haemolytic dilutions, immediately haemolyse when alkalis/

alkalis are added, or where Co_2 is bubbled through the fluid: if on the other hand the alkali be added first and the soaps later, a retardation of haemolysis occurs.

Ponder investigated this phenomenon independently, and found the same to be true of saponin and bile salts, when acids or alkalis were added. Neutral substances produced little effect. The degree of acceleration of the haemolysis depends on the amount of acid or alkali added: adding small amounts may produce a retardation. There seems little doubt that these changes are concerned with surface tension alterations. Ponder (27) showed that saponin adsorbs alkalis, according to the equation

$$\frac{x}{m} = aC^{\frac{1}{n}}$$

the letter C denoting concentration of alkali, x the amount adsorbed, in the quantity of adsorbent, and a and $\frac{1}{n}$ being constants. The value of $\frac{1}{n}$ is about 3; it has been shown the $\frac{1}{n}$ in adsorption equations is always between 2 and 10. This adsorption may influence the action of the saponin: the non-adsorbed acid will further affect the cell. It seems possible that it weakens the cell wall, for the order in which acids act as accelerators is that in which they themselves haemolyse. The haemolytic phenomena which occur when/

When these complex agents are employed to produce haemolysis are not easily analysed. They differ markedly when cells which are not freshly prepared are used, but such cells are more resistant to haemolysis than fresh cells: similarly cells prepared in normal NaCl are more resistant than cells prepared in citrated saline. The explanation of this may be found in the presence of calcium, this substance diffusing out of the erythrocytes after they have stood for some time: Calcium would render the membrane impermeable relatively speaking, and so delay haemolysis by a substance which acted by virtue of its low surface tension.

XXVI. It is probable, however, that the accelerating effect of small quantities of various substances is not due to an injury, of a permanent nature, to the cell wall, for the accelerating effect of an acid which has been in contact with cells for some time may be neutralised by the addition of suitable quantities of a base, and vice versa. The change in the cell is physical and not chemical, if there be a change at all: the acceleration may, however, be due to an increased action of the saponin because of adsorbed acid or alkali.

XXVII. One of the chief difficulties which has confronted many investigators in the explanation of how a substance can take several hours to produce haemolysis by means of/

of a surface tension effect; the prevalent idea being that the substance produces a sudden lowering of surface tension at the red cell interface, as soon as added to the fluid containing the cells, and that this sudden stress ruptures ~~the~~ the cell. Several observers have remarked that surface tension changes would account, no doubt, for a rapid haemolysis, but not for a slow one, extending over some hours. This remark is based on a misconception of what actually must occur. When such a substance is added to a suspension of erythrocytes, each cell is subjected to a stress, tending to drag its envelope in an outward direction. Such a stress can never produce haemolysis unless the envelope yields, and becomes stretched. This yielding requires time, because it is determined by the rate at which fluid can permeate the membrane, passing into the cell, and thus, by allowing the volume to increase, allowing a stretch of the capsule. Once the envelope begins to yield, - which it will do, not at its circumference, but at the centre of the disc - this part will become the weakest part of the envelope, and therefore there will the stress have most effect. The progressive yielding of the envelope will depend on the rate at which fluid can pass into the cell, and this again will depend on (I) the permeability of the membrane and (II) on the outside stress.

If/

If the stress be small, there is no reason why the process should not take hours.

D. HAEMOLYSIS REGARDED AS A RESULT OF DESTRUCTION
OF THE CELL ENVELOPE.

XXVIII. The simplest way to explain the breaking up of the cell is to regard the breaking as a result of destruction of the cell wall. This destruction may be due to chemical action of a haemolytic substance, or to solubility of the components of the envelope in the haemolytic agent. Injury to the cell wall may be caused by freezing, as is well known, and haemolysis may result: haemolysis may be produced by injury, as shown by Guthrie (49), or by abstracting water, as with glycerol, according to Simon (50). Hot saline will haemolyse red cells, by wetting their envelopes; but Tokaki states that saline from 48^o-70 °C will not melt the envelope, but yet causes haemolysis, presumably by removing certain components of the envelope only. (51). Haemolysis may be produced by electric shocks: how far this is due to direct injury, and how far to ionisation of the neighbouring/

neighbouring fluid, is uncertain.

XXIX. Many observers attribute all forms of haemolytic action to ~~gross~~ injury to the cell, excepting the haemolysis produced by hypotonic saline. Bechhold and Kraus (52) detected three stages in the haemolysis of cells by corrosive sublimate, using the ultramicroscope. First the substance was concentrated at the cell interface, then a disintegration of the membrane was observed, and finally the contents of the cell were seen to be protruded in bubble-like processes: the corrosive sublimate appeared to have disintegrated the membrane. Some substances undoubtedly do this: saponin and bile salts both dissolve lipoids, and so may weaken the cell envelope; the extent to which they do this, is, however, probably small (XXIV). Schrender (53) thinks that saponin unites with some component of the cell: it is known to unite with cholesterol, to form an insoluble compound. Arrhenius considers that saponin combines with the proteins. Haffner and Jodlbauer (54) producing haemolysis by various dyes, consider a destruction of the cell membrane to be responsible for the haemolysis. Rohonyi used chlorates, nitrites, and ferricyanides as haemolytic agents; he believes that the cell adsorbs the substance, which disintegrates the cell: the idea of a membrane is rejected/

rejected. (55). Some substances are certainly adsorbed according to the adsorption law, - for example, rose bengal, a dye. (54). Rohonyi's arguments are, however, very unsound indeed: he makes no attempt to make his statements agree with the work of others. He considers osmosis has little or nothing to do with haemolysis by chemical substances, the adsorption of which he speaks being independent of osmotic pressure. He considers the erythrocyte to be a solid body of a colloidal nature. Neilson and Wheeldon also consider the red cells to be "jellies", which are haemolysed by destruction, which is produced by various substances. The lecithin component of these jellies is supposed to be able to unite with substance, forming toxic *lecithides*: such *lecithides* disintegrate the cell, by a process similar to plasmolysis. (56).

XXX. The β -rays of radium have been shown by Hausmann (57) to produce haemolysis: presumably by injury to the envelope, since these rays are injurious to protoplasm. Possibly, however, they ionise the surrounding fluid, and produce haemolysis by osmosis.

E. HAEMOLYSIS BY "ANIMAL HAEMOLYSINS".

XXXI. The action of certain haemolysins of animal origin, but of unknown composition, will now be considered.

It is intended to omit the consideration of haemolysins which require activation by complement, since the consideration of such comes under part F. Under this heading falls the haemolysins secreted by the cobra, scorpion, bee, spider, and by various snakes.

Various snakes, especially the cobra, produce a highly haemolytic poison. This poison may be dried and kept either in glycerol, or as a dry powder. It haemolyses red cells of all species; when these cells are washed free of serum, some species, notably the horse, offer a considerable resistance, but in the presence of serum, horse cells are haemolysed also. The presence of inactivated serum is said to protect the cell, to some extent (Lunz and Gyotgy, 58): this is not remarkable, since red cells are protected in this way against the action of many substances, for example, bile salts (Ponder, 59). The explanation of the action of the venom is given by Kridicke and Sachs (60) and by Houssay and Negrette (61). Cobra venom hydrolyses lecithin and nucleoproteins; thus it attacks the cell envelope. This hydrolysis is aided by the presence of calcium. Haemolysis by the venom has been shown to be accelerated by calcium. It is sometimes stated/

stated that by the hydrolysis, a highly haemolytic lecêthide is produced, and that this lecêthide is thermolabile: the postulation of the existence of such a substance appears unnecessary; a hydrolysis of lecêthin and protein in the cell envelope is sufficient to account for the haemolysis: the evidence for the presence of the lecêthide is that, if the hydrolysis continue, the lecêthide itself is destroyed, and haemolysis ceases. It appears equally reasonable to assume that the hydrolysis ceases after a certain time, owing to the venom combining with the degeneration products.

XXXII. The scorpion poisons are secreted by glands in the last joint of the scorpion's tail. Iwano finds that they hydrolyse into leucine, tyrosin, lecethin, and cholesterol. They are haemolytic: pepsin and trypsin destroy their haemolytic power, perhaps by hydrolysing them. (62). They do not haemolyse the cells of the rabbit, cat or goat (Mori, 63). Judging from the figures given by Mori, their haemolytic action is not very great. Mori's results regarding species are a little doubtful, since ~~this~~ technique is faulty. The mode of action of the scorpion poisons is unknown.

XXXIII. The bee secretes^e a haemolysin. This substance hydrolyses into lecêthin, tryptophane, palmitic acid, butyric acid, and a saponin. (Flury, 64).

Presumably/

Presumably it acts similarly to saponins, but since Flury had to extract poison from 200,000 bees in order to get sufficient to analyse, little work has been done on the haemolytic activity of the substance.

XXXIV. The *Epeira* family of spiders produce a haemolytic toxin: the females alone produce it, but it is contained in the eggs. Little is known about it (Levy, 65). The poison was once considered to be a protective one; it is haemolytic only to the erythrocytes of mammals, however, and even then not strongly so.

F. HAEMOLYSIS BY AMBOCEPTOR-COMPLEMENT SYSTEMS.

XXXV. Most of the work on amboceptor complement systems has not been carried out with the object of elucidating the mode of action of haemolysins, but has been concerned rather with complement fixation by immune bodies. It has been known for a long time that, if one animal be injected with suspensions of blood cells of another animal of a different species, the injected animal develops in its blood a haemolysin, specific for the blood cells of the second animal.

This

This haemolysin will only act in the presence of complement. The haemolysin plus complement thus forms a amboceptor - complement system. Certain animals have the haemolysin naturally in the blood: for example, human serum contains a haemolysin for sheep-cells.

XXXVI. The red cells of the animals whose blood is injected acted as an antigen: the nature of antigen is unknown, but antigens are inseparable from proteins, in most cases. The lipoids of the injected erythrocytes are not antigens: there is scarcely any evidence that lipoids can act as antigens; injections with lipoids calls forth no immune body. It may therefore be taken that the antigen is the protein component of the erythrocyte.

XXXVII. Just as little is known regarding the nature of the immune haemolysin. It is destroyed by heating to about 70°; it is non-dialisable, and appears to be a protein. Its action is specific; it haemolyses only the cells of that species whose cells acted as antigen: this is not always true however, as a rabbit injected with guinea-pig's kidney will develop a haemolysin for sheep's erythrocytes. (Forssman 66). McDonagh considers that it is a lipid-globulin, (67), with the same stereo-chemical composition as the lipid-globulin in the injected erythrocytes. According to Landsteiner/

Landsteiner and Jagic, colloidal silicic acid can replace it. (68): but silicic acid is not specific as is an immune haemolysin.

XXXVIII. The way in which the immune haemolysin combines with complement, has been a subject for much speculation: but there is little actual knowledge regarding it. The immune haemolysin will not act without complement: no true substitute for complement has yet been found, although much effort has been made (Von Liebermann and Von Fennyvessy, 69). Lipoids cannot replace complement; peptone, however, can act as an activator, under certain circumstances. (Lampe, 70). Complement behaves in a peculiar way: it seems to be very unstable, and the researches of Azzi and Porcelli-Totone have suggested that "complement" is not a definite substance at all, but merely a "condition" under which haemolysin can be part of a physical system of a certain type. Azzi inactivated complement by shaking (71): Porcelli-Totone succeeded in subjecting the complement in dry serum to a temperature of 100° without injuring it. (72).

XXXIX. The manner in which complement haemolysin produces haemolysis is unknown. Attempts have been made to correlate the activity of such systems with their surface/

surface tension: Schmidt has shown that no inference can be drawn from the surface tension of a haemolytic serum (73). There seems no doubt that the haemolysin produces some change in the red cells: for cells subjected to the haemolysin without complement are rendered susceptible to the haemolytic action of soaps. (69). Clowes (74) suggests that the antigen-antibody complex affords a link between water and a non-aqueous phase, which exists in the erythrocyte wall, whereby the latter becomes suddenly permeable, and allows haemoglobin to diffuse through it. Jobling and Bull (75) found that an immune haemolysin with or without complement, produced a breaking down of the lipoids in the envelope of the red cell: this they considered to be the mechanism of haemolysis. McDonagh (67) thinks that the red cells "adsorb the amboceptor", thus "altering osmotic pressure", and causing liberation of haemoglobin. The views of this observer are however worthy of little attention, since they are based on pure speculation, and since it is obvious that he uses terms without a clear conception of their meaning.

XL. It may be concluded that the mode of action of amboceptor-complement systems is still unknown; but that probably they act by disintegrating the cell envelope. Arrhenius (76) considers that the action can be explained in/

in a strictly quantitative way, as chemical reactions can be explained. He considers that the cell envelope is attacked chemically, and broken down; complement and amboceptor unite and form a compound which effect this. Neither act merely as "catalysts" or sensitisers". Where a = cc of amboceptor, b = c.c. of complement, x , the amount of haemolysin produced, in one case investigated was given by the equation,

$$(5a - 2) (20b - x) = 90x.$$

Doubt has been cast on these results.

MATHEMATICAL INVESTIGATIONS.

G

XLI.

It is possible to investigate haemolysis from a more general point of view than any discussed in the previous sections. Regardless of whether it be the result of osmosis, surface tension, or cell destruction, one may attempt to measure accurately various factors, and to express relations between them, hoping thereby to arrive at generalisations. Since in the production of haemolysis by any substance there are many factors involved, such as temperature, concentration, amount of blood haemolysed, etc., in order to make measurements, each of these factors must be controlled, and their effect on a single uncontrolled factor in the reaction observed. Which of the factors shall be uncontrolled is largely a matter of taste. There are, however, two methods which are used largely; - the first is to find the quantity of haemolytic agent which will produce certain haemolytic effects in a given time, while the second is to observe the time taken for a certain quantity of agent to effect haemolysis. In the first method the dependent variable is quantity, in the second, time. The first method/

method has the advantage that it avoids the introduction of certain rather complicated equations expressing velocity relations: when the velocities are not uniform, these may lead to difficulties. The second method, however, has the advantage that the time may be measured with great accuracy, the "trial and error" method which working to a given time end-point involves being avoided. It also seems more reasonable to leave time, over which one has no control, a dependent variable, than to arrange a reaction so as to be complete in a given time, - often a very difficult and tedious operation. Further in reactions of variable velocity it is fallacious to limit the reaction in this way.

XLII. It has been found that the phenomena of haemolysis may be expressed by certain definite formulæ, which hold to within the limits of experimental error. The first investigation of any degree of completeness was made by ARRHENIUS (76), who showed that the law of mono-molecular reactions applied to haemolysis.

The law of monomolecular reactions states that the curves representing the logarithm of the quantity of the haemolysed substance, as a function of time, is a straight line. The velocity of the reaction at any moment is expressed, therefore,

$$\frac{\partial x}{\partial t} = k(a - x) \quad \text{or} \quad k = \frac{1}{t} \log \frac{a}{a-x}.$$

Where a is the initial concentration of substance to be haemolysed, x the amount transformed in the time t , and k a constant depending on the nature of the reacting system:- the "constant of velocity".

Further, ARRHENIUS showed that the qt rule applied to haemolysis. This rule stated that half the quantity of haemolytic substance requires double the time to produce the same result. The qt rule applies, according to ARRHENIUS to many vital phenomena, as well as to haemolysis.

XLIII

The effect of temperature on haemolytic reactions was then investigated. Rise of temperature accelerates haemolysis, as it accelerates most processes. The influence of temperature on reactions in general, is given by the formula:-

$$K_1 = K_0 e^{\frac{\mu}{T_1} (T_1 - T_0)}$$

where T_0 and T_1 are the two temperatures in absolute units, K_0 and K_1 the respective velocities at T_0 and T_1 and μ a constant. If T_0 and T_1 are close to one another, the value $T_0 T_1$ does not alter much in the interval, and the formula becomes:-

$$K_1 = K_0 e^{c(T_1 - T_0)} \quad \text{or,} \quad \log K_1 - \log K_0 = c(T_1 - T_0).$$

Therefore, ~~log~~ K is nearly a linear function of the temperature becoming/

becoming greater as the temperature rises.

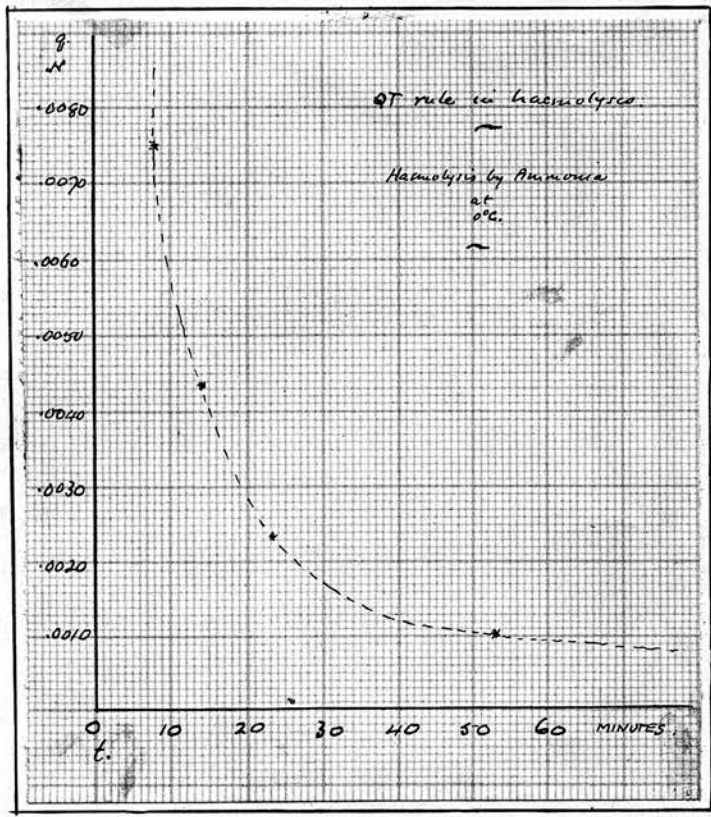
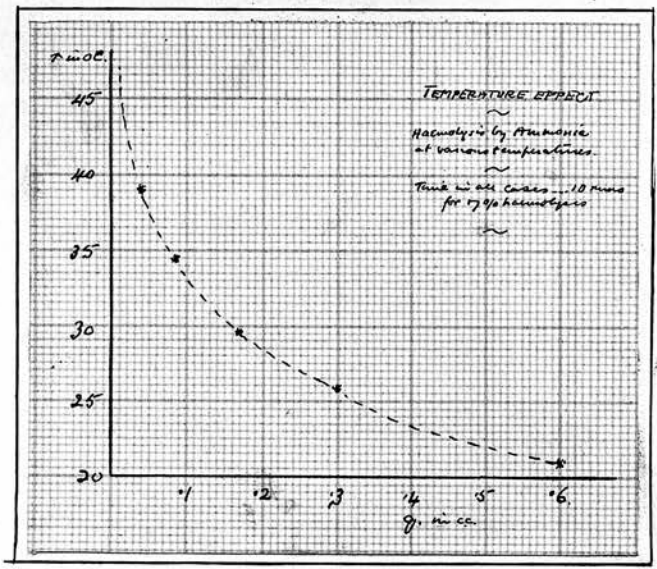
ARRHENIUS found that this formula described very well the velocity of haemolysis by hot water, the value of μ being 64,200, and also haemolysis by means of acids, bases and lysins, where μ varied from 25,000 to 30,000. He found, observed and calculated results to agree closely in the majority of cases. Certain exceptions were observed, however, notably sodium oleate. In these investigations the quantity of reacting substance was varied so as to give a haemolysis in a fixed time.

When the qt rule is introduced into consideration of velocities at various temperatures, one finds that it may be stated that if a fourfold quantity is necessary for reaching the same haemolytic effect at 29°C. as at 39°C. one may say that the same quantity of haemolytic substance would occupy four times as long to produce the same effect at 29°C. as at 39°C.

XLIV

Some examples will now be given to make these points clear. Using a 5 per cent suspension of cells, and haemolysing by means of ammonia at 0°C. results were obtained as expressed in the following table. The end point chosen was 40% haemolysis.

Concentration/



CONCENTRATION of AMMONIA	TIME for HAEMOLYSIS t.	VALUE of PRODUCT qt.
0.001 N.	53 mins.	53
0.00237 N.	23 mins.	52.21
0.00435 N.	14 mins.	60.9
0.0075 N.	8 mins.	60.0

The product qt is thus nearly a constant. The experimental difficulties in this case are great, since partial haemolysis is a bad end point.

The relation between q and t is obviously expressed by a rectangular hyperbola, the value of k being variable with the temperature.

The temperature effect may be illustrated by the following example, which concerns the haemolysis of red cells by ammonia, in 0.5 N. solution, at different temperatures. The quantity of ammonia required to haemolyse 8 c c of a 1 per cent suspension of cells in 10 minutes is denoted by q . τ is the temperature in degrees C. The end point was 17 per cent haemolysis.

τ	q	q calc.
21	0.6	0.64
25.9	0.3	0.30
29.7	0.17	0.17
34.8	0.085	0.083
39.5	0.04	0.043

The value of μ from which the calculated results were obtained is 26,760: these results correspond closely with observed ones.

XLV

ARRHENIUS has not expressed the relation between t and τ ; - i.e. between the time taken by a quantity of haemolytic substance to produce its effect, and the temperature at which the experiment is conducted: - but this relation may be deduced in the following way.

The relation between τ and q is expressed by a hyperbola approaching, as an asymptote, the straight line $q=0$. The equation for this curve is therefore,

$$\tau = \frac{\alpha}{\beta}(\beta - q) + \frac{\gamma}{q}.$$

where α , β and γ are constants. Or, simplifying,

$$\frac{\alpha}{\beta}q^2 - \alpha q + \tau q - \gamma = 0$$

But the relation between q and t is,

$$qt = k, \quad \text{or} \quad q = \frac{k}{t}.$$

Therefore,

$$\frac{\alpha}{\beta}\left(\frac{k}{t}\right)^2 - \frac{\alpha k}{t} + \frac{\tau k}{t} - \gamma = 0.$$

Or, what is the same thing,

$$\frac{\alpha k^2}{\beta} - \alpha kt + \tau kt - t^2 \gamma = 0.$$

Dividing by K throughout, and rearranging,

$$\tau t = -\frac{\alpha k}{\beta} + \alpha t + \frac{\gamma t^2}{k}.$$

Now, /

Now, since $kt = k$, in one case at least $k = t^2$.

Therefore substituting t^2 for k ,

$$\tau t = \frac{-at^2}{\beta} + at + \delta$$

or

$$\tau = \frac{a}{\beta} (\beta - t) + \frac{\delta}{t}.$$

which is an equation relating τ and t , and which is represented by a hyperbola approaching the straight line $t = 0$ as an asymptote. This important deduction will be referred to later.

XLVI

ARRHENIUS further investigated the resistance of erythrocytes. When a volume of blood cell suspension is haemolysed by a substance, certain of the cells are haemolysed before others. The time taken for the haemolysis of each cell is in ratio to its resistance, the weak cells going first, the strong ones last. The mean resistance might be expected to be halfway between the extremes; that is, the distribution of the erythrocytes as regards resistance will be in accordance with the law of probability. This was demonstrated to be the case. (77). This observation is not new, for the same conclusion might be arrived at from the consideration of well enough known data. Pasteur-Vallery, Radot, and ~~Sh~~érítier showed that the resistance of erythrocytes varied as their size in direct proportion (78). Now Malassez (79) showed that the size of erythrocytes varies in distribution, according to the law of probability: Malassez did/

did not apparently realise that he had demonstrated this, but such a conclusion may be arrived at from his figures. Accordingly we would expect that the resistance varied also according to this law.

XLVII. Many of ARRHENIUS' statements have been challenged by other observers, and his formulae shown in many cases to be merely first approximations. This is, in particular, true of the investigations on haemolysis. PONDER (80) has found fault with some of the formulae, particularly with those involving the qt rule. He raises two objections to ARRHENIUS' methods (a) that the end-point used was a percentage haemolysis, and not a complete haemolysis, observation being therefore very difficult, and a possibly non-uniform reaction being stopped before completion, and (b) that by making quantity instead of time the dependent variable, room for large errors is introduced. In order to avoid these errors, a special technique was introduced, whereby the end-point of haemolysis could be got with great accuracy, and where the velocity of reaction was measured, instead of the quantities taken to produce a degree of haemolysis previously arranged. A number of chemical substances which possessed haemolytic properties were examined including, Saponin/

Saponin	Acetic acid
Sodium glycocholate	Citric acid
Sodium taurocholate	Benzoic acid
Lactic acid	Ammonium chloride

It was found possible to describe the action of these substances by formulae, more accurate than those of ARRHENIUS.

XLVIII. It has been seen (section XLV) that by ARRHENIUS' formulae the relation between time and temperature for the action of a haemolytic substance should be.

$$\tau = \frac{\alpha}{\beta} (\beta - T) + \frac{\delta}{T}$$

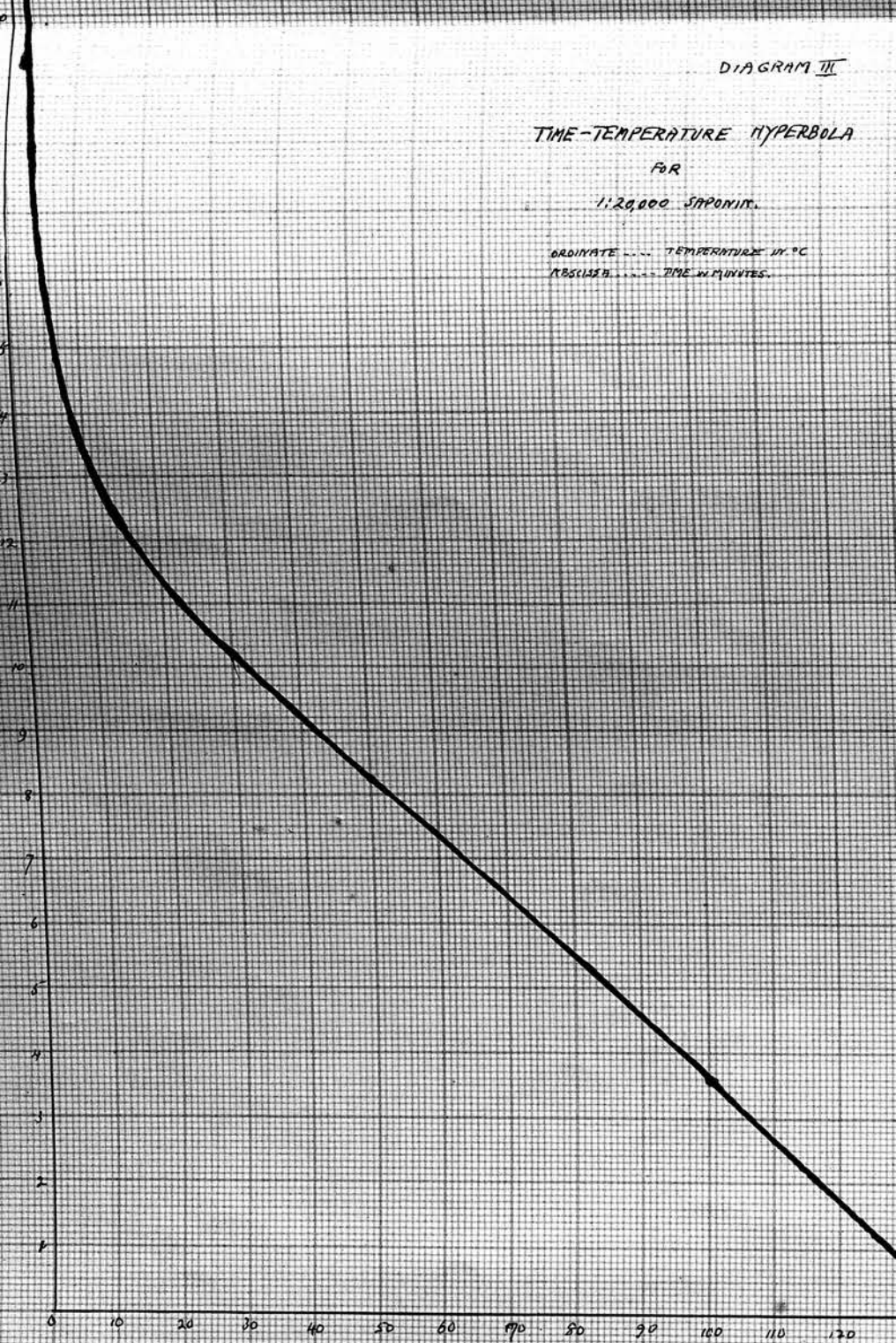
where T = the time in minutes taken for the reaction, τ = the temperature at which the reaction takes place, and where α , β , and δ are constants. This equation is that of a hyperbole, one of whose asymptote is the straight line $T = 0$.

Such a relation will be found to exist. The constants α , β , and δ are readily found, for α is the intercept on the τ axis, and β the intercept on the T axis, if the part of the hyperbole in the neighbourhood of 1° C. be considered/

DIAGRAM III

TIME-TEMPERATURE HYPERBOLA
FOR
1:20,000 SAPONITE.

ORDINATE TEMPERATURE IN °C
ABSCISSA TIME IN MINUTES.



considered a straight line; δ can be found from the equation, T and τ being known. The following table and diagram III.,

give an example for saponin, in a dilution of 1 in 20,000, acting on 1 cc of standard suspension (cells in saline) at various temperatures from 0 to 40 C. The values of the constants in this case are $\alpha = 12.4$, $\beta = 139$, $\gamma = 16.6$;-

τ	T
0	139
3.6	100
8.2	50
10.2	30
20.5	2
28.8	1
40.	.5

This general time-temperature relation was found to hold in all cases.

XLIX. The haemolytic activity of any substance in a given dilution obviously depends on the constants which apply to that dilution. There must then be a relation between the values of each constant and the dilution, for any haemolytic substance/

substance.

Taking first the constant α , it was found that it varied with δ the number of cc which contained 1 gramme of the haemolytic substance in the following way,

$$\alpha = m\delta + n.$$

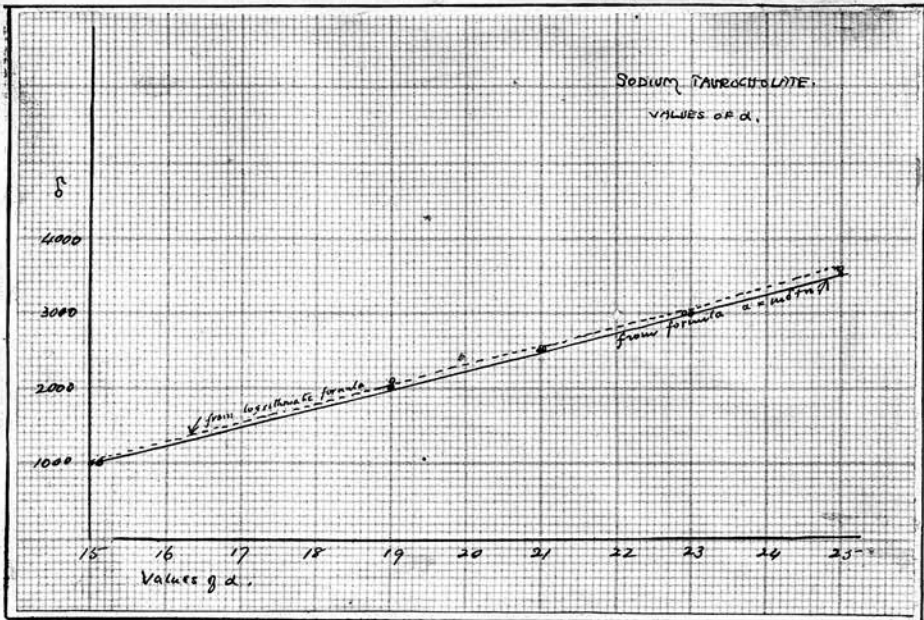
where m and n are constant for any haemolytic substance. This is the equation of a straight line, and the smallest value of α would be n . Various theoretical considerations regarding the activity of very active haemolytic systems, whose constants can be found by extrapolation, have led to a modification of this formula, which may be expressed by saying that $\log \alpha$ is a linear function of $\log \delta$ or, in general terms,

$$\alpha = K\delta^{\frac{1}{k}}$$

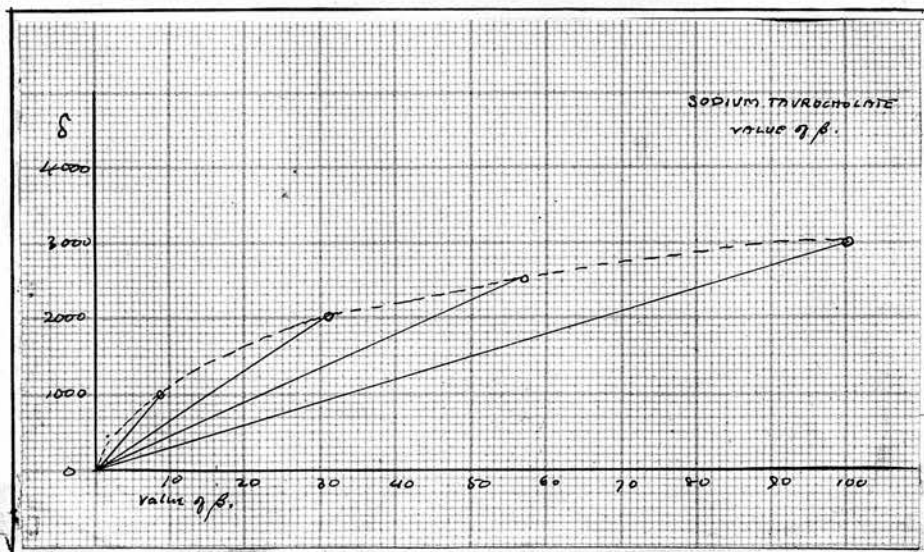
K and $\frac{1}{k}$ being constants for any haemolytic substance. The linear equation is a very good approximation to this for such values of α as have a measurable haemolytic activity; the degree of approximation is shown in diagram. IV. a.

Now considering the constant β , it will be found that it varies with δ in a somewhat complex way. If plotted against/

a.



b.



against values of δ , a curve results, as seen in diagram.

IV, b. which gives the β values of ^{SOD. TAUROCHOLATE.} ~~saponin~~. If points on the curves, p_1, p_2, p_3 , etc., corresponding to values of $\delta_1, \delta_2, \delta_3$, etc., be joined to the origin $\beta=0, \delta=0$, a series of angles $\theta_1, \theta_2, \theta_3$, result between the joining lines and the abscissa. These angles, expressed in degrees, are related to δ by a hyperbole, approaching the line $\theta=0$ as an asymptote, and expressed therefore by the equation.

$$0.01\delta = \frac{a}{b}(6-\theta) + \frac{c}{\theta}.$$

the relation of β to δ is therefore,

$$0.01\delta = \frac{a}{b}(6-\theta) + \frac{c}{\theta} \dots\dots\dots (i).$$

$$0.01\delta = \beta \tan \theta \dots\dots\dots (ii).$$

a, b, and c being constants. The value of b will be the intercept on the θ axis, that of a the intercept on the δ axis: c can be calculated. The value of b is very stationary: theoretically it can vary between an infinitely small value and 90. In the cases of all substances of measurable activity it has been found to be approximately 65; in certain very active substances whose constants are determinable by extrapolation it approaches 90.

The constant δ is linear with δ , and is given by the/

the equation,

$$\delta = p\delta$$

p being a constant.

Thus if the values of a, b, c, p, k, and $\frac{1}{\lambda}$ are known the velocity of reaction under any conditions of temperature or dilution may be calculated.

L. Since at 0° C, $\beta = T$, according to the qt rule, at this temperature

$$q\beta = k \quad \text{or} \quad \beta = k\delta.$$

since q is the reciprocal of δ . But

$$\beta \tan \theta = \delta, \text{ so } \beta \tan \theta = \frac{1}{q} \text{ and } \beta = \frac{1}{q \tan \theta}$$

Therefore,

$$k\delta = \frac{1}{q \tan \theta} \text{ or } k = \frac{1}{\tan \theta}$$

from which it follows that

$$qt = \frac{1}{\tan \theta}$$

Now $\frac{1}{\tan \theta}$ is not a constant, but varies with q. When q and t are equal, $\theta = 45^\circ$ and $\frac{1}{\tan \theta} = 1$; small differences of q will accordingly cause only small differences of $\frac{1}{\tan \theta}$, so that within certain limits $\frac{1}{\tan \theta}$ may be looked upon as constant, and it may be wrongly assumed that the qt rule is of general application/

application. Since ARRHENIUS investigated only comparatively small ranges of dilutions, it is easy to see why he concluded that the reaction was described by the qt rule.

- LI. The haemolytic activity of a substance depends chiefly on the manner in which β varies with ρ , if the reaction proceed at 0 °C, if at a higher temperature, on the variations of α and β with ρ . Since the value of α depends chiefly on the value of m ,— or $\frac{1}{n}$ in the revised equation,— and the value of β chiefly on the value of the constant a , it is probable that there is a relation between a and m .

$$\text{Let } \frac{a}{h} = \tan \phi.$$

$$\text{and } 100m = \tan \omega.$$

then we have the relation,

$$0.75\omega^2 + \omega(\phi - 45^\circ) - 52 = 0$$

Similarly,

$$n(90 - \omega) = 750,$$

so that n , m , a , and $\frac{1}{h}$ are all interdependent. Further c , and p are interdependent, for

$$c = 500 p.$$

Thus all the constants are linked up. This is not surprising, for it must be remembered that when it is said that a substance produces haemolysis, it merely means that the red cell gives way before a certain stress. The stress on the cell which causes it to burst is constant/

constant, and in all haemolytic experiments it is the velocity with which this stress is attained that is being measured. Consequently if one can determine the velocity for this occurrence in one case, where a particular dilution is concerned, the question comes to be how the dilution affects the velocity. To assume such relations as have been given above is, then, merely equivalent to saying that the time taken to perform a certain reaction varies with the amount of substance involved in performing that reaction, in a similar way, whatever that substance be, provided that it acts at all. The intensity of its action is described by the values of its constants. An example of such a law is the qt rule, which assumes that q multiplied by t gives a constant: this constant will be small for an active substance, great for an inactive one. The above formula also say that there is a relation between q and t - or in other symbols, between δ and T - but that the relation is not nearly so simple. There is, however, no difference in principle, and indeed, it has been shown in sections XLV and L that the qt rule is an approximation to the more extensive formulae, which tells one that at 0° C, instead of $qt = k$ and t or $T = \delta k$, the value of T is,

$$\frac{a\theta - \frac{a}{f}\theta^2 + c}{\tan \theta \cdot \theta}$$

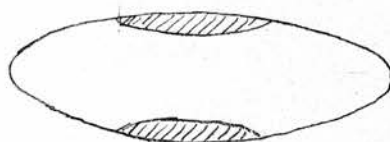
LII. Since the erythrocyte, if it be a cell with an elastic membrane, will become larger in volume if water diffuses into it, the envelope thereby being stretched and eventually bursting, it is important to know in what way such entry of water will affect its shape, volume, and the area of the envelope. This has not hitherto been investigated, as the evolution of the necessary formulae are very difficult. Very interesting results appear, however, if these matters be studied.

PONDER (27) has worked out formulae by which the behaviour of the erythrocyte under internal pressure may be studied. The cell is circular on horizontal section, and in vertical section is biconcave. It may be regarded, with very little error, as a large ellipsoid of rotation about its minor axis, from which two smaller similar ellipsoids are subtracted. This is seen in diagram **V** : the body is seen in vertical section. The area enclosed in the red line is an ellipse, if the two small shaded ellipses be subtracted, the biconcave form of the red cell results.

Now the formula for the volume of an ellipsoid of rotation may be arrived at as follows:-

Let the figure in diagram **V** be an ellipse, with the semi-axis minor = B , and the semi-axis major = A . Consider
a/

DIAGRAM V.



a strip at a distance x from the axis minor, and let the height of this strip above and below the axis major be y .

Then A , the area of the figure will be

$$\int_0^a 2y dx.$$

and the volume V of the ellipsoid will be

$$\begin{aligned} & \int_0^a 2y dx \cdot 2\pi x. \\ &= 4\pi \int_0^a yx dx. \\ &= 4\pi b \int_0^a \sqrt{1 - \frac{x^2}{a^2}} x dx. \end{aligned}$$

since

$$\frac{x^2}{a^2} + \frac{y^2}{b^2} = 1.$$

and

$$y = b \sqrt{1 - \frac{x^2}{a^2}} \dots \dots \dots (i)$$

To integrate

$$\int \sqrt{1 - \frac{x^2}{a^2}} x dx$$

let $\sqrt{1 - \frac{x^2}{a^2}} = \sin \theta$, then $\cos \theta = \frac{x}{a}$,

and $x = a \cos \theta$. so $dx = -a \sin \theta d\theta$.

Thus/

Thus we get,

$$\begin{aligned} & -a^2 \int \sin \theta \cos \theta \sin \theta \, d\theta. \\ & = -a^2 \int \sin^2 \theta \cos \theta \, d\theta. \dots \dots \dots (ii) \end{aligned}$$

leaving out $-a^2$,

$$\begin{aligned} & = \sin^2 \sin \theta - 2 \int \sin \theta \cos \theta \, d\theta. \\ & = 3 \int \sin^2 \theta \cos \theta \, d\theta. \\ & = \frac{1}{3} \sin^3 \theta. \end{aligned}$$

Then substituting this value, in (ii),

$$\frac{a^2}{3} \sin^3 \theta.$$

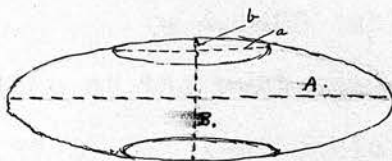
Substituting in (i),

$$\begin{aligned} V &= 4\pi b \int_0^a -\frac{a^2}{3} \left(1 - \frac{x^2}{a^2}\right)^{\frac{3}{2}}. \\ &= \frac{4\pi b a^2}{3}. \end{aligned}$$

LIII. Using the formula the volume of the erythrocyte may be calculated to a close approximation.

If we have a large ellipsoid of rotation about its minor axis, B and A representing the semi-minor axis, and the major semi-axis respectively, and if from it we subtract/

subtract two small ellipsoids with semi-axes b and a , then the remaining part (figure) will be approximately the shape of the red cell.



If we assign to A , B , a and b suitable values, the volume of the shaded figure may be calculated.

Since $2A$, the diameter of the erythrocyte = 7.8μ

$$A = 3.9 \mu$$

and since the greatest thickness = 2μ

and " least " = 1.5μ

$$2b = 0.5 \mu$$

$$\text{and } b = 0.25 \mu$$

Further, $2B$ = the least thickness + $4b$

$$2B = 2.5 \mu$$

$$\therefore B = 1.25 \mu$$

The value of a is difficult to determine, but, judging from averages from microphotographs,

$$a = 2 \mu$$

Then the volume of the large figure is,

$$\frac{4\pi A^2 B}{3}$$

$$= 79.5872 \text{ cubic } \mu$$

and/

and the volume of each small ellipsoid is,

$$\frac{4\pi a^2 b}{3}.$$

$$= 4.1888 \text{ cubic } \mu$$

Therefore the volume of the part shaded is,

$$79.5872 - 8.3776 = 71.1096 \text{ cubic } \mu.$$

The average volume of the erythrocyte may thus be taken at 71.1096 cubic μ : the error is very small.

LIV.

It is of great importance to know how an ellipsoid of rotation will behave under internal pressure, for this will give a key to the behaviour of the erythrocyte when fluid diffuses into it. An ellipsoid with an extensile wall of equal extensibility throughout, will tend, if the pressure inside be raised, to assume a form in which the pressure is equal at all points of the wall. It is a well-known fact that the pressure is greatest where the curvature is least: accordingly the centres of the discs will yield first, and the body will eventually become a sphere - for a sphere is that form in which internal pressure is equally distributed. The ellipsoid will become such a sphere by a bulging of its sides, the minor axis of the ellipse lengthening, and the major axis growing no greater, but, indeed, becoming somewhat less: ultimately the increasing minor axis will equal the/

the major axis, and a sphere will result. If the pressure be further raised, the radius of the sphere will increase. Applying this to the erythrocyte it may be concluded that:-

- (i). The volume of the biconcave disc increases by a bulging of the sides. It will be obvious that the biconcave disc will become an ellipsoid of rotation if volume

$$\frac{4\pi A^2 B}{3}.$$

(where $2A$ = the diameter of the cell, $2B$ = the smallest thickness + 4 times the difference between the greatest and the smallest thickness), without any stretching of the envelope. Taking average cells, it can increase from a volume of about 70 cubic μ to a volume of about 80 cubic μ , without any stress of the envelope.

- (ii). If diffusion continues B will increase, while A remains constant, or decreases exceedingly (negligibly) little. The increase in B will continue until $B = A$, which is the condition for a sphere. Now the internal pressure will be equally distributed, and the volume contained in the envelope will be

$$\frac{4\pi R^3}{3},$$

R being the radius and $A = B$.

In/

In the average cell again, this volume would be 288 cubic μ .

- (iii). If the internal pressure be still raised R will increase until the cell bursts.

LIV. It is possible to make accurate measurements of the diameter of red cells from microphotographs. This diameter measurement is of great importance, since under certain circumstances, it is possible to calculate several factors from it such as, the breaking strain of the envelope. There are two ways of approaching the problem: one is to photograph individual cells under various conditions, while the other is to deal with averages.

(1) To find the degree of stretching of which the envelope is capable, in a given cell, it is necessary to photograph the cell, under a magnification of about 1150x, first in isotonic saline, and then in saline which causes haemolysis. This is possible, but difficult. If, however, a cell transferred from normal saline to .45 per cent sodium chloride, and photographed immediately after the transfer, has a diameter of 7.9μ , while after being for some time in this hypotonic saline its diameter increases to 8.2μ we may say that its volume has increased from about 70 cubic μ to 288 cubic μ : if at this point haemolysis occurs, it is a simple matter to find the increase in length of a strip of the envelope passing through points of greatest stress. The length of the strip subjected to the greatest extension will be, in this case

$$2\pi \sqrt{\frac{3.9^2 + 1.25^2}{2}} \mu.$$

before stretching begins, while at the point of haemolysis the length of the strip will be

$$2\pi \times 4.1 \mu.$$

The actual measurement of diameter can be made on a number of cells, the volume and extension calculated, and an average arrived at, which will give the degree of stretching which the envelope will withstand before bursting.

(II) The measurement may be made on large numbers of cells. 100 cells are photographed in isotonic saline. Their diameters are measured, the mean and standard deviation found. Using Malassez' figures (79), the mean diameter is found to be 7.49μ and therefore σ , the standard deviation is 1.046. The probable error of the mean is .07, as 100 cells are considered. Other series of photographs are taken, of cells of the same individual, in hypotonic saline, after various lengths of time: haemolysed cells, as "ghost corpuscles" may also be photographed. For each series the mean diameter and the standard deviation, etc., is calculated: the result of the investigation is to give a series of diameters, statistically correct, for cells in various hypotonicities of saline, after various periods. From these diameters, volumes and envelope extension may be calculated as before: provided the mean diameter/

diameter of a series exceeds the mean diameter of the cells in isotonic saline. The method accordingly gives us ^{no} indication of the early changes produced, but only of changes in volume when the cell is considerably swollen and nearing haemolysis.

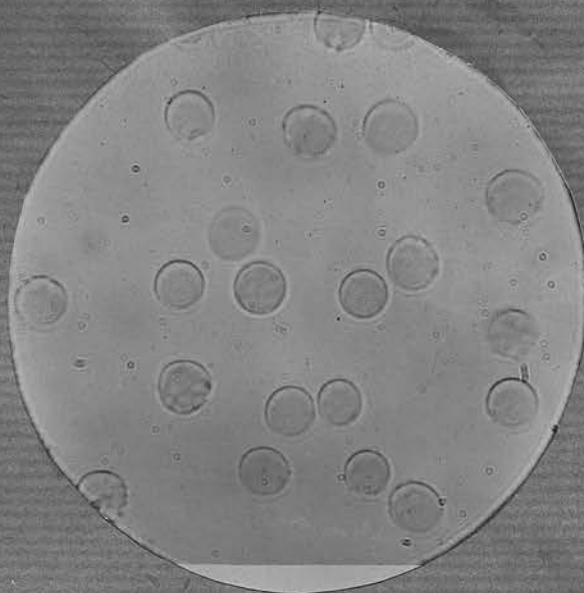
Taking an actual experiment, the following results were obtained. The cells used were haemolysed by .4 per cent sodium chloride. Several fields of about 20 cells were taken, and the results treated statistically.

	Mean Diameter μ	σ
Cells in isotonic saline	7.3 \pm .062	0.31
" " hypotonic " 5 mins.	6.48 \pm .064	0.34
" " " " 15 "	7.4 \pm .056	0.29
" " " " 30 "	8.15 \pm .047	0.17

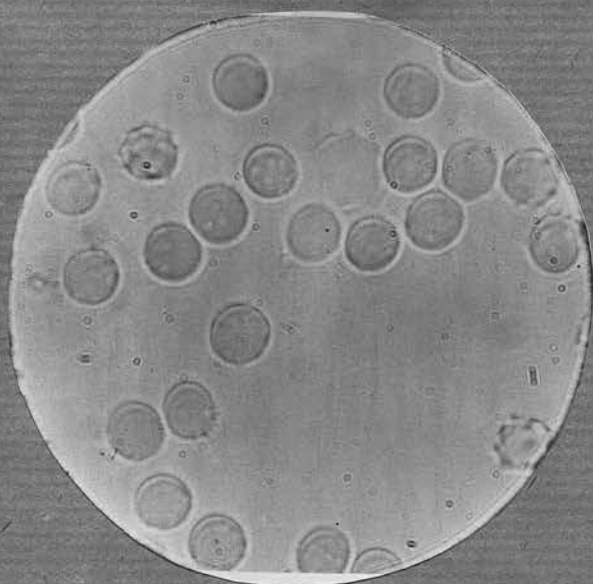
These prints of negatives from which these measurements were made are seen in Figure VI. The magnification is 1000x. The measurements were made from the negatives which were obtained by using Wratten plates, and a green colour filter.

It will be seen that (a) first the average diameter decreases, (b) afterwards, when a sphere is formed, the radius increases, (c) nearly all cells are haemolysed when their diameter is 8.1 μ ; after an increase in diameter of about .8 μ .

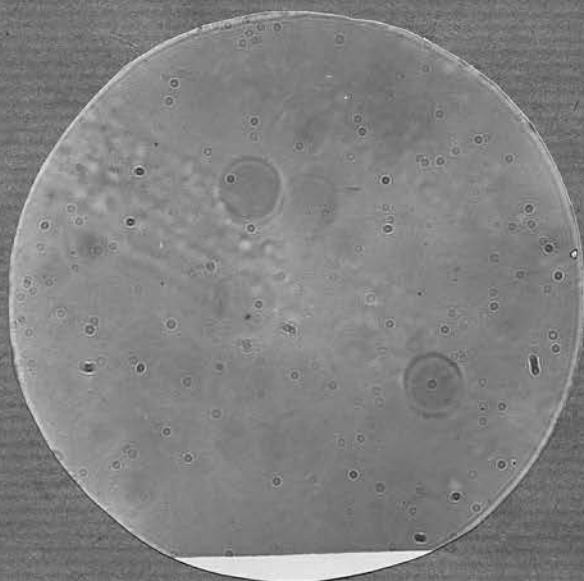
INITIAL STAGE ... DIAMETER SHORTENED.



SECOND STAGE, SPHERES.
DIAMETERS SAME AS
IN UNREPTED CELLS



FINAL ... CELLS HAEMOLYSED.
DIAMETER INCREASED



LV. The statement of Price-Jones (4), that the red cells increase by as much as $.6 \mu$ during the day, etc., seems to be at variance with these results. It remains for Price-Jones to explain, how in a body of the nature of the erythrocyte, this increase can occur: for it is obvious from the preceeding sections, that such an increase would imply that such cells were so swollen as to be spherical, further an average increase of $.6 \mu$, or anything approaching it, would be accompanied by haemolysis of many cells. Doubtless the error into which this writer has fallen is simply that of assuming that an average obtained in the usual way, is an infallible guide. Mallassez's figures show a σ of 1.046μ , and a probable error of $.07 \mu$. Thus an increase in average diameter of less than $.21 \mu$ would be of no significance. This statistical error, together with the far worse one of making measurements from stained films, explain the extraordinary statement of Price-Jones.

LVI. The applicability of this method involving the diameter measurement is very great. Taking the case of alkalis, for instance, it may be shown that during haemolysis by these substances, a considerable increase of diameter of the cells occur. This could not be accounted for by solubility of the envelope; but probably implies a negative surface-tension stress on the envelope; Also in the case of saponin and bile salts, this increase of diameter occurs, undoubtedly due to an outward drag on the envelope, due to lowering of surface tension. Solubility of the cell wall would not account for such an occurrence.

Since/

Since this method is only newly evolved, full details for these haemolytic substances cannot be given: the general method, however, is that indicated in the example given above.

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